Journal of Apicultural Research and Bee World 47(4): 286–291 (2008)

DOI: 10.3896/IBRA.1.47.4.09

ORIGINAL RESEARCH ARTICLE

X-ray computerised microtomography (MicroCT): a new technique for assessing external and internal morphology of bees.

Mark Greco[1], Allan Jones[2], Robert Spooner-Hart[1] and Paul Holford[1*]
[1]Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia.
[2]Australian Key Centre for Microscopy and Microanalysis, Madsen Building, F09, University of Sydney NSW 2006, Australia.

Received 4 February 2008, accepted subject to revision 13 April 2008, accepted for publication 14 May 2008.

*Corresponding author. Email: p.holford@uws.edu.au

Summary

Traditionally, the classification of bees has been conducted with the aid of dissecting (light) microscopy. In more recent times, detailed information on external and internal morphology for bee classification has been obtained using scanning and transmission electron microscopy. MicroCT is emerging as a new method for non-invasive 3D computerised X-ray tomographic imaging of insects at the microscopic level and, in this study, has been evaluated for its use in morphological studies of bees. A Skyscan 1172 MicroCT system was used to assess the internal and external morphology of the Australian stingless bee *Trigona carbonaria* with particular focus on the proventricular plates. MicroCT was useful in non-invasively visualising gross external morphological features such as the articulations of the coxae, trochanters, tibiae and tarsi of each leg including broadened hind basitarsi. Image magnification revealed further detail such as antennal scapes and the various parts of the tongue including the proboscis and labium. However, the individual facets of the eye were barely discernable and MicroCT did not reveal fine details of hairs on the body or legs. Internal morphology was clearly visualised, including the tracheal system and details of the proventriculus and proventricular basal plates which form the leaflets of the proventricular valve. Thus, the characteristic features of the proventricular basal plates of Meliponini could be quickly and easily identified non-invasively. Therefore, MicroCT, as one of the emerging techniques of diagnostic radioentomology, has particular advantages for non-invasively and non-destructively imaging bees and, particularly, rare or more scientifically valued insects such as museum specimens and those trapped in amber.

Microtomografía computerizada de rayos X (MicroCT): una nueva técnica para evaluar la morfología externa e interna de las abejas.

Resumen
Tradicionalmente, la clasificación de las abejas se ha realizado con la ayuda de microscopios de disección. En tiempos más recientes se ha obtenido información detallada de la morfología externa e interna de las abejas usando microscopia de transmisión de electrones y escaneado. MicroCT se está convirtiendo en un método nuevo de toma de imágines mediante tomografía de rayos X computerizada 3D no invasiva al nivel microscópico y, en este estudio, se ha evaluado su uso en estudios morfológicos de abejas. Se ha usado un sistema Skyscan 1172 MicroCT para evaluar la morfología externa e interna de la abeja sin aguijón australiana *Trigona carbonaria* enfocándose en las placas proventriculares. MicroCT ha sido útil en visualizar no invasivamente partes de la morfología externa burdas como las articulaciones de los trocánteres de las coxas, y la tibia y el tarso de cada pata incluyendo los basitarsos posteriores más anchos. La ampliación de las imágenes reveló otros detalles como las partes de la antena y varias partes de la lengua incluyendo la probóscide y el labio. Sin embargo las facetas de los ojos apenas se distinguieron y MicroCT no reveló detalles finos de los pelos del cuerpo o de las patas. La morfología interna se visualizó más claramente incluyendo el sistema traqueal y detalles de las

placas basales proventriculares y del proventrículo las cuales forman las dos mitades de la válvula proventricular. Entonces las partes más características de las placas basales proventriculares de los Meliponini pueden ser rápida y fácilmente identificadas de forma no invasiva. Por tanto MicroCT en una técnica emergente de diagnóstico radioentomológico que tiene ventajas particulares para la toma de imágenes de abejas de forma no invasiva ni destructiva, y particularmente, para insectos raros o de un valor científico especial como los especímenes de museos o aquellos atrapados en ámbar.

Keywords: MicroCT, stingless bees, morphology, proventricular plate, diagnostic radioentomology

Introduction

Bees evolved from among the apoid wasps at least 60 million years ago (Melo, 1999; Engel, 2000; O'Toole and Raw, 2004) and the gathering of information for their appropriate classification continues because they are not yet formally ranked as a monophyletic group under current classification by melittologists (Melo and Gonçalves, 2005). Current classification places bees in the superfamily Apoidea along with wasp families such as the sphecidae (Brothers, 1999; Melo, 1999), but with increases in molecular data as a basis for their classification and as new diagnostic imaging techniques arise, a definitive classification of bees as a single group is emerging (Engel, 2000; O'Toole and Raw, 2004; Grimaldi and Engel, 2005; Melo and Gonçalves, 2005). Apart from the necrophagic *Trigona hypogea* (Noll, 1997), bees are pollen-feeding, aculeate Hymenopterans (Michener, 2000). They exist as solitary bees, semi-social bees or in eusocial colonies (Michener, 1974). They have characteristic morphologies such as branched or plumose body hairs, broadened hind basitarsi and many species have cleft claws (Michener, 2000). Roig-Alsina and Michener (1993) suggested that all stingless bees belong to one tribe, the Meliponini. This hypothesis has been supported by Serrao (2001) who looked at the proventricular morphology of bees and determined that the Euglossini and Bombini have long columnar proventricular plates, the Apini have triangular apices to their plates whilst the Meliponini have slender and elongated plates. Thus, the proventriculus is an important diagnostic structure for bee taxonomy.

Traditionally, the morphological classification of bees has been conducted with the aid of dissecting light microscopy (Michener, 1960; Wille, 1979) to visualise structures such as wing venation, tarsal segments and other morphology. In more recent times, detailed information for bee classification based on their external and internal morphology has been obtained with the use of scanning electron microscopy (SEM) (Serrao, 2001; Serrao, 2005) and transmission electron microscopy (TEM) (Araujo *et al*., 2005). The data from Serrao (2001) suggest that the Meliponini and Apini are a monophyletic group and that Bombini and Euglossini are also a monophyletic group. Although SEM and TEM provide the highest level of detail, sample preparations are laborious, time consuming, invasive and destructive for each investigation (Serrao, 2001).

X-ray computerised tomography (CT) has previously been adopted to visualise macroscopic characteristics of insects. MicroCT is now emerging as a new method for the non-invasive imaging of insects at the microscopic level (Hornschemeyer *et al*., 2002; Johnson *et al*., 2004). The basic principles of MicroCT are similar to those used in medical CT scanners; however, with MicroCT, it is now possible to achieve a resolution down to a few micrometers (Bettuzzi *et al*., 2004; Feeney *et al*., 2006). In this paper, we describe the use of MicroCT for non-invasively and non-destructively assessing the morphology and anatomy of an Australian stingless bee, *T. carbonaria*, with particular focus on the proventricular plates.

Materials and methods

Sample preparation

For MicroCT, a worker of the bee species *T. carbonaria* was placed in a 0°C freezer for 15 minutes. *Trigona carbonaria* was selected for examination as it is relatively common and well studied (Michener, 1961, 1974; Heard, 1988a, 1988b, 1994, 1999, 2001; Bartareau, 1996; Dollin *et al*., 1997; Dollin, 1998, 1999, 2000; Amano *et al*., 2000; Amano, 2002, 2004; Greco *et al*., 2005). The dead bee was placed in a 15 mm length of polyethylene drinking straw (internal diameter 3 mm). The two ends of the straw were sealed with polystyrene foam to keep the bee in position whilst being scanned. For light microscopy, a worker bee was dissected and viewed using a Leica MZ12 stereomicroscope, Leica Microsystems GmbH Ernst-Leitz-Strasse 17-37 35578 Wetzlar. For comparison of internal abdominal morphology, a pinned, thirteen-year-old *Amegilla* sp. was also scanned using the same preparation as above; however, the specimen was not dissected afterwards for preservation reasons.

MicroCT scanning

Scans were performed with a Skyscan 1172 high-resolution MicroCT system. This system has a sealed, microfocus x-ray tube with a 5 µm focal spotsize. The x-rays were produced by exposing the anode to 40 kV at 100 µA. Prior to scanning, the sample was placed on the pedestal between the x-ray source and the CCD detector. After positioning the sample, 600 2D x-ray images over 180° were captured by exposing the sample and then rotating it to the next exposure position with a slice-to-slice rotation distance of 2 µm, and a total acquisition time of approximately 60 min: each 2D image represents one slice. The scanner software then converted each slice to axial orientation and created 998 bitmap images (16 bit grey scale) which were stored for 2D viewing and 3D reconstruction as a 983Mb dataset.

Image processing

The 3D reconstruction and analysis of the x-ray images were performed using VG STUDIO MAX V1.2 voxel data analysis and visualization software (Volume Graphics GmbH; Germany) and BeeView 3D visualisation software (Disect Systems Ltd; Suffolk, UK). Using their multi-planar reformatting (MPR) algorithms, these software programs enabled the 2D x-ray images to be

reformatted into a 3D model. The model was further manipulated by adjusting window levels (WL) and window widths (WW) to enhance visualisation of the morphological structures. Greatest visual enhancement was achieved at WL 150 and WW 158. For greater flexibility in software usage, VG STUDIO MAX was used while at the scanner workstation and BeeView was used remotely on a laptop computer. The sample was viewed from many angles along randomly selected axes. Sections of the model were also removed along cutting planes which were positioned by using the computer mouse. The cutting planes acted like a virtual scalpel, enabling visualisation of the bee's internal morphology including fine detail of the bee's proventricular plates. Image magnification was performed when greater detail was required.

Results

Although the resolution of MicroCT is not as fine as SEM, the images presented in this paper demonstrate that the technique is useful for viewing and assessing the external and internal morphology of bees accurately and non-invasively. Gross external morphological features such as the articulations of the coxae, trochanters, tibiae and tarsi of each leg including broadened hind basitarsi (Fig. 1) could be seen. Image magnification revealed further detail such as antennal scapes and the various parts of the mouth including the proboscis and labium (Fig. 2). However, the individual facets of the eye were barely discernable and MicroCT did not reveal fine details of hairs on the body or legs.

The two software programs (VG STUDIO MAX V1.2 and BeeView 3D) were user-friendly and provided powerful image processing, including the ability for virtual dissection. Unfortunately, many soft tissues did not produce sufficient differentiation, and it was not possible to discern structures such as ovaries and details of the hind gut. Attempts were made to use medical grade CT contrast agent to outline these soft tissue regions; nevertheless the results were very poor and more research into the use of CT contrast agents will be required to improve soft tissue differentiation in insects using MicroCT. However, some internal structures could be clearly visualised. For example, Fig. 1d shows the lateral longitudinal tracheal trunk. In particular, because the proventriculus and proventricular plates are lined with cuticle, these structures were better differentiated and were clearly visualised and discernible for diagnostic purposes (Figs 3 & 4). Fig. 3b shows the slender and elongated outlines of the basal plates that line the proventriculus and which form the leaflets of proventricular valve. The leaflets of these plates are covered in hairs (Serrao, 2005), but these were not visible using MicroCT. Figs 3c and d show the proventriculus at the distal part of the crop and the cross-shaped leaflets of the proventricular valve forming the opening into the proventriculus. Fig. 4 shows images of the proventriculus using dissection light microscopy and MicroCT. The image acquired from light microscopy resulted from dissections of six worker bees which were destroyed in the process whereas the images acquired during MicoCT required just one bee which was undamaged. The characteristic features of the proventricular plates of Meliponini could thus be quickly and easily identified non-invasively. Fig. 5 demonstrates the type of morphological variations that will be identified in more detailed future studies using MicroCT. The internal morphology of the fifth abdominal sternite of these very different bee species is similar, but the *T. carbonaria* sternite shows a pronounced notch along its lateral margin which is absent in the *Amegilla* species.

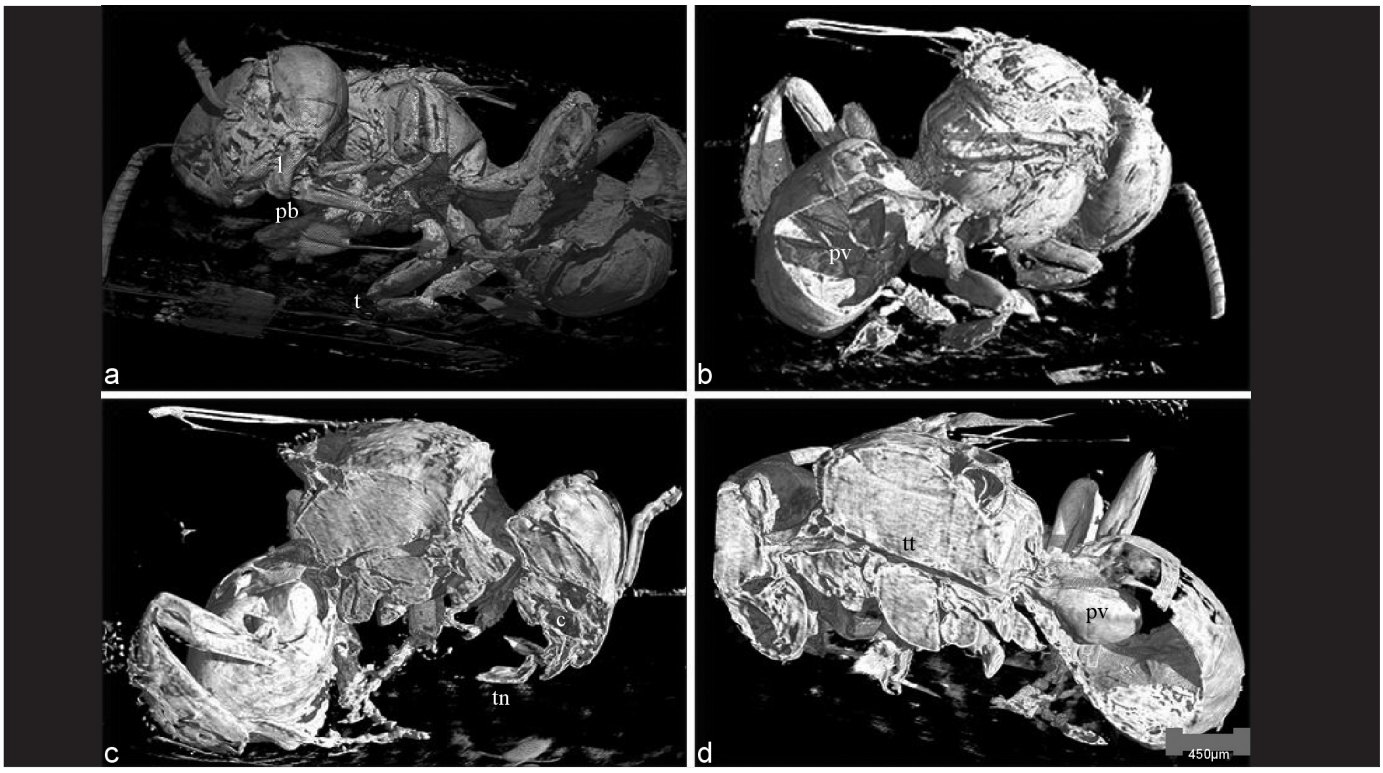

Fig. 1. MicroCT images of a worker bee of *T. carbonaria*: **a.** ventral aspect showing tarsi (t), proboscis (pb) and labium (l); **b.** postero-dorsal view with virtual dissection of abdomen and proventriculus (pv); **c.** right lateral view showing cibarium (c) and longitudinal section of the tongue (tn); and **d.** left lateral view showing lateral longitudinal tracheal trunk (tt) and proventriculus (pv). The images were processed using VG STUDIO MAX.

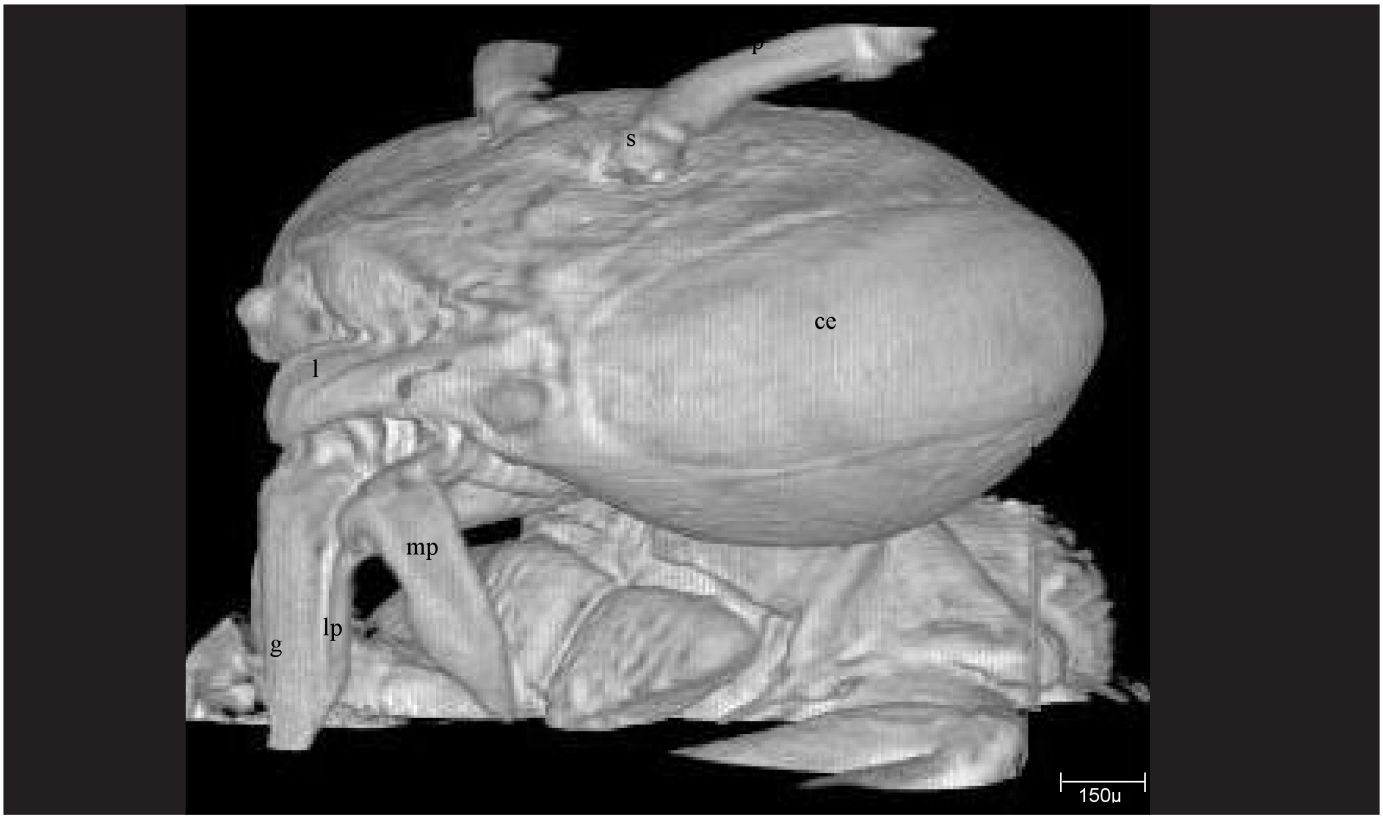

Fig. 2. 3D reconstruction of the head of a worker of *T. carbonaria* showing details of external morphology: antennal pedicle (p), antennal scape (s), compound eye (ce), labrum (l), maxillary palpus (mp), labial palpus (lp) and glossa (g).
The image was processed using VG STUDIO MAX.

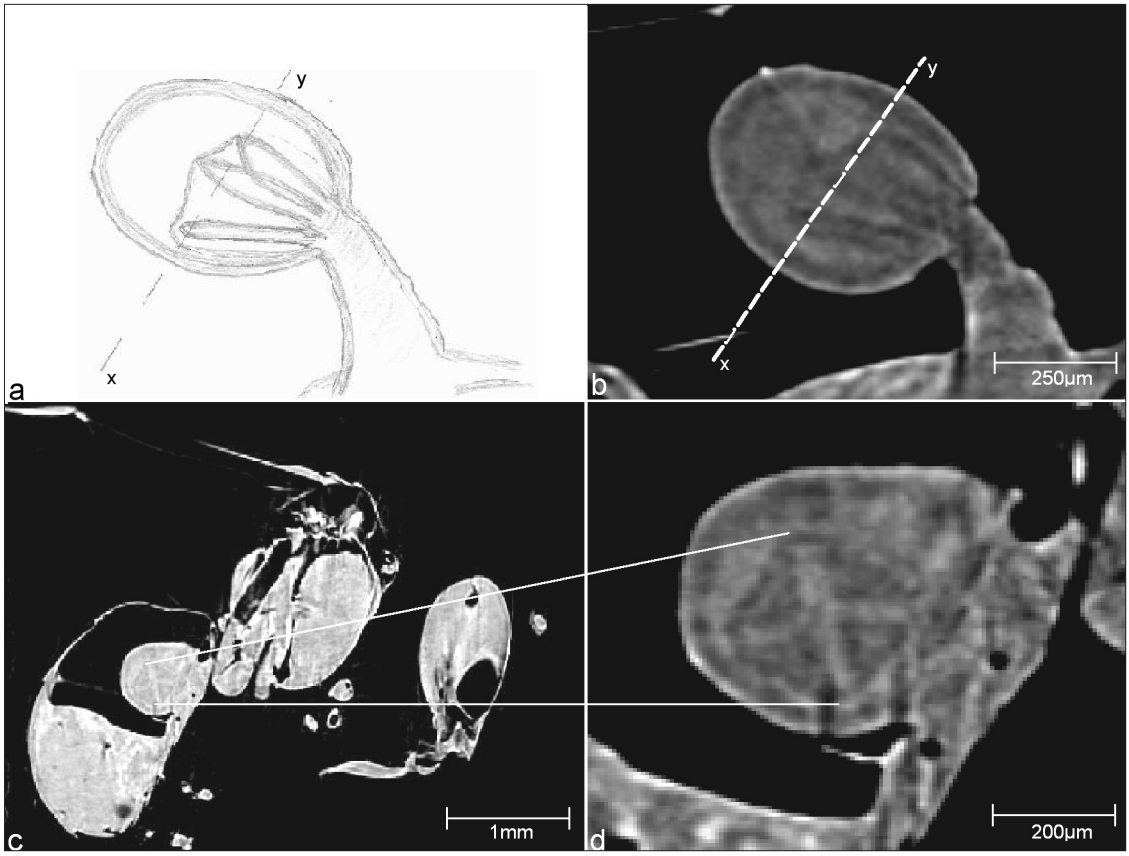

Fig. 3. Details of the proventriculus, proventricular valve and elongated proventricular plates of *T. carbonaria*: ***a***. line diagram of the image in Fig 3b; ***b***. cross section of proventriculus showing characteristic slender, elongated, Melliponini-shaped plates using MicroCT; ***c***. MicroCT of proventricular valve showing the four plates in closed position; and ***d***. magnified image after using electronic dissection at cutting plane x-y. The images were processed using BeeView.

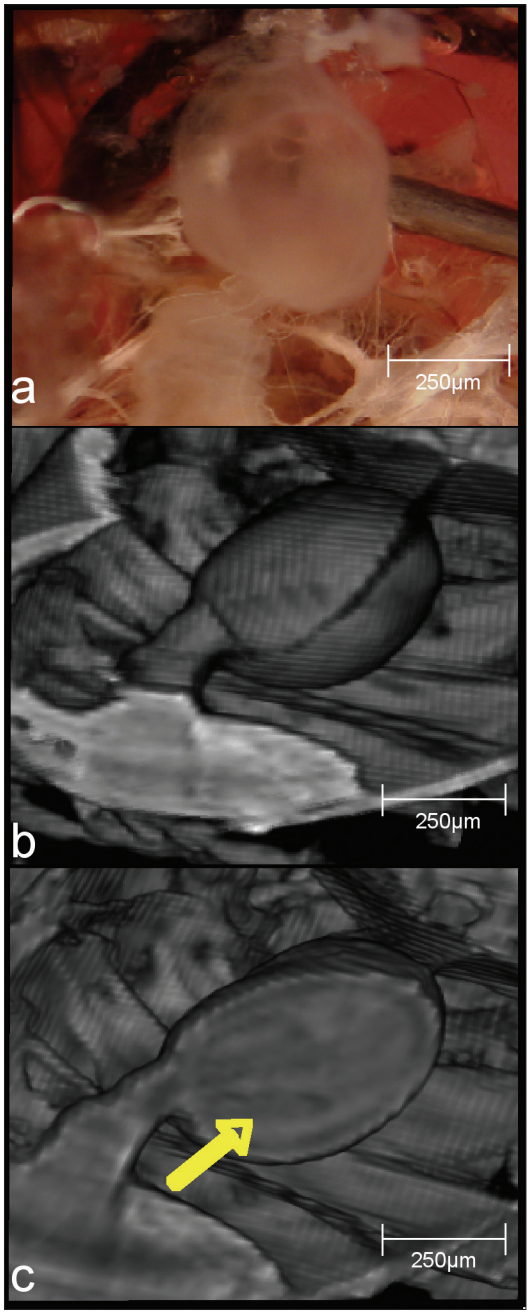

Fig. 4. a. Dissection light microscopy of *T. carbonaria* showing the proventriculus. ***b., c.*** MicroCT images of the proventriculus showing: ***b.*** a 3D reconstruction of proventriculus and ***c.*** details of the slender, elongated, proventricular plates (arrowed). The images were processed using BeeView.

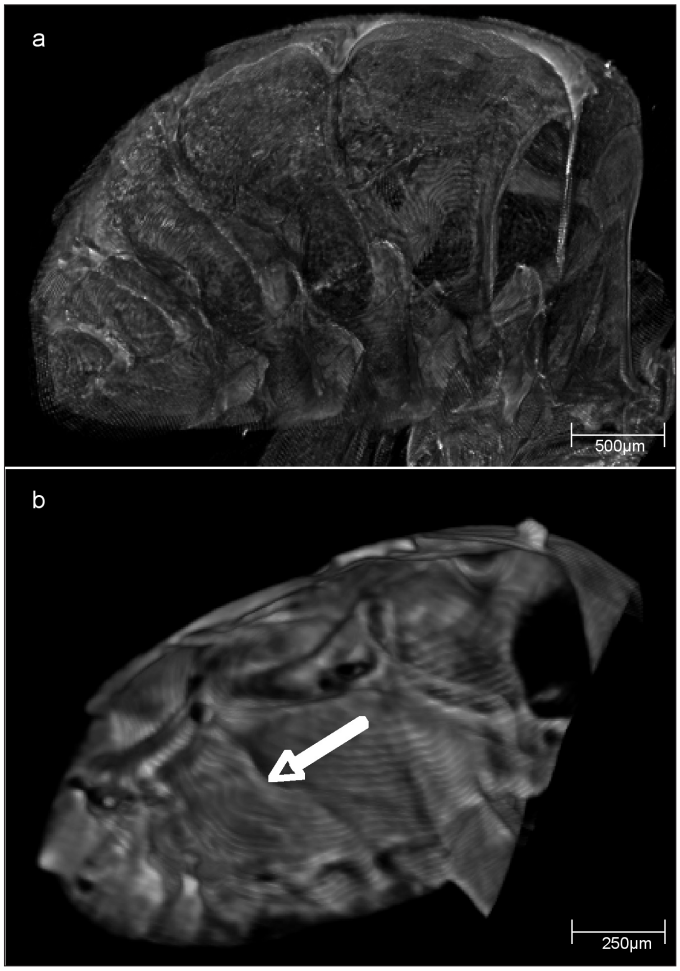

Fig. 5. 3D MicroCT images showing internal abdominal morphology of two very different bee species. ***a.*** A thirty-year-old, museum specimen of an *Amegilla* sp. showing the internal morphology of the sternites, and ***b.*** a fresh specemin of *T.carbonaria* showing similar morphology but with a notch evident on the lateral margin of the fifth sternite (arrow). The images were processed using BeeView.

Discussion

This new approach is not destructive, unlike the traditional methods of dissection light microscopy, SEM or TEM (Serrao, 2001; Araujo *et al*., 2005; Serrao, 2005). The procedures outlined in this paper allowed the visualisation of internal and external morphological features to be carried out without lengthy and laborious dissections or preparations required by these techniques. Although the samples in this study were dead, the scans could also be performed on live, immobilised insects without harming them. The scans take approximately 1 h and once the images are saved as electronic datasets in bmp, jpeg or DICOM formats, the 3D reconstruction and virtual dissecting can be repeated indefinitely. In addition, virtual dissection can be performed in planes at infinite angles on the same specimen which cannot be done when using SEM, TEM or light microscopy, thus making morphological analysis much less laborious for the researcher. The researcher can also electronically store each dataset and return to the same sample at a later stage to review the same morphology or explore new regions within the sample.

The non-destructive nature of MicroCT will be invaluable for rare or precious specimens, such as those in museums, where there may be only one or two specimens available for examination, or for scanning holotypes. MicroCT also has the advantage of being able to scan insects in amber or as fossils

without damaging the specimens. This will have important uses for non-invasive phylogenetic studies on ancient insects such as bees in amber.

MicroCT, along with NanoCT and MacroCT, which we collectively describe as "diagnostic radioentomology", are emerging technologies (Tollner, 1991; Fuchs *et al*., 2004; Greco *et al*., 2005, 2006) that will prove useful for entomologists. The recent refinements in NanoCT imaging will allow image resolution to move closer to the detail revealed by SEM, making diagnostic radioentomology the technique of choice in the future.

Acknowledgements

The scanning for this study was performed at the Australian Key Centre for Microscopy and Microanalysis at the University of Sydney, part of the Nanostructural Analysis Network Organisation (NANO) and we would like to thank the staff of this unit for their scientific and technical assistance. The authors would also like to thank Michael Batley for donating the *Amegilla* specimen.

References

DONOVAN, B J; REID, P E C (1988) The alfalfa leafcutting bee, *Megachile rotundata* (Megachilidae), does not pollinate kiwifruit, *Actinidia deliciosa* var. *deliciosa* (Actinidiaceae). *Journal of Apicultural Research* 27(1): 9–12.

AMANO, K (2002) Stingless honey bees for Asia's greenhouses. *Food & Fertilizer Technology Center* [online] http://www.fftc.agnet.org/library/article/nc138a.html. (accessed on 11 January 2008).

AMANO, K (2004) Attempts to introduce stingless bees for the pollination of crops under greenhouse conditions in Japan. *Food & Fertilizer Technology Center* [online] http://www.fftc.agnet.org/library/article/tb167.html. (accessed on 11 January 2008).

AMANO, K; NEMOTO, T; HEARD, T A (2000) What are stingless bees and why and how to use them as crop pollinators? *Japan Agriculture Research Q.* 34: 183–190.

ARAUJO, V A; ZAMA, U; DOLDER, H; LINO-NETO, J (2005) Morphology and ultrastructure of the spermatozoa of *Scaptotrigona xanthotricha* Moure (Hymenoptera: Apidae: Meliponini). *Brazilian Journal of Morphological Science* 22: 137–141.

BROTHERS, D J (1999). Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta* 28: 233–249.

BARTAREAU, T (1996) Foraging behaviour of *Trigona carbonaria* (Hymenoptera: Apidae) at multiple-choice feeding stations. *Australian Journal of Zoology* 44: 143–153.

BETTUZZI, M S; CASALI, F; CORNACCHIA, S; ROSSI, M; PALTRINIERI, E; MORIGI M P; BRANCACCIO, R; ROMANI, D (2004) A new linear array detector for high resolution and low dose digital radiography. *Nuclear Instrumentation and Methodology* B 213: 227–230.

DOLLIN, A. (1998) Stingless beekeeping with *Trigona* in Vietnam. *Aussie Bee* 7: 7–9.

DOLLIN, A (1999) A winter's tale… Stingless bees thrive in glasshouse trial. *Aussie Bee* 12: 8–11.

DOLLIN, A (2000). Stingless bees pollinate greenhouse capsicums. *Aussie Bee* 13: 15.

DOLLIN, A; DOLLIN, L; SAKAGAMI, S (1997) Australian stingless bees of the genus *Trigona* (Hymenoptera: Apidae). *Invertebrate Taxonomy* 11: 861–896.

ENGEL, M S (2000) A new interpretation of the oldest fossil bee (Hymenoptera: Apidae). *American Museum Novitates* 3296: 1–11.

FEENEY, D S; CRAWFORD, J W; DANIELL, T; HALLETT, P D; NUNAN, N; RITZ, K; RIVERS, M; YOUNG, I M (2006) Three-dimensional microorganization of the soil–root–microbe system. *Microbiology and Ecology* 52: 151–158.

FUCHS, A; SCHREYER, A; FEUERBACH, S; KORB, J (2004) A new technique for termite monitoring using computer tomography and endoscopy. *International Journal of Pest Management* 50: 63–66.

GRECO, M K; BELL, M; SPOONER-HART, R; HOLFORD, P (2006) X-ray computerized tomography as a new method for monitoring *Amegilla holmesi* nest structure, nesting behaviour and adult female activity. *Entomologia Experimentalis et Applicata* 120: 71–76.

GRECO, M K; SPOONER-HART, R; HOLFORD, P (2005) A new technique for monitoring *Trigona carbonaria* nest contents, brood and activity using X-ray computerised tomography. *Journal of Apicultural Research* 44: 97–100.

HEARD, T A (1988a) Establishment and propagation of hives of the stingless bee *Trigona carbonaria*. *Proceedings of the Second Australian and International Beekeeping Congress* pp 114–116.

HEARD, T A (1988b) Propagation of hives of *Trigona carbonaria* Smith (Hymenoptera: Apidae). *Journal of the Australian Entomological Society* 27: 303–304.

HEARD, T A (1994) Behaviour and pollinator efficiency of stingless bees and honey bees on macadamia flowers. *Journal of Apicultural Research* 33: 191–198.

HEARD, T A (1999) The role of stingless bees in crop pollination. *Annual Review of Entomology* 44: 183–206.

HEARD, T A (2001). Stingless bees and crop pollination. *Bee World* 82: 6–7.

HORNSCHEMEYER, T; BUETEL, R G; PASOP, F (2002) Head structures of *Priacma serrata* Leconte (Coleoptera, Archostemata) inferred from x-ray tomography. *Journal of Morphology* 252: 298–314.

JOHNSON, S N; READ, D B; GREGORY, P J (2004) Tracking larval insect movement within soil using high resolution X-ray microtomography. *Ecological Entomology* 29: 117–122.

MELO, G A R (1999) Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on the crabronid wasps. *Scientific Papers, Natural History Museum of the University of Kansas* 14: 1–55.

MELO, G A R; GONÇALVES, R B (2005). Higher-level bee classifications (Hymenoptera, Apoidea, Apidae *sensu lato*). *Revista Brasileira de Zoologia* 22(1): 153–159.

MICHENER, C D (1960) Observations on the behaviour of a burrowing bee (*Amegilla*) near Brisbane, Queensland (Hymenoptera, Anthophorinae). *The Queensland Naturalist* 16: 63–67.

MICHENER, C D (1961) Observations on the nests and behaviour of *Trigona* in Australia and New Guinea (Hymenoptera: Apidae). *American Museum Novitates* 2026: 1–46.

MICHENER, C D (1974) *The social behaviour of the bees: a comparative study.* Harvard University Press; Cambridge, USA.

MICHENER, C D (2000) *The bees of the world*. John Hopkins University Press; Baltimore, USA.

NOLL, F B (1997) Foraging behaviour on carcasses in the necrophagic bee *Trigona hypogea* (Hymenoptera: Apidae). *Journal of Insect Behaviour* 10(3): 463–467.

O'TOOLE, C; RAW, A (1991). *Bees of the world.* Blandford; London, UK.

ROIG-ALSINA, A; MICHENER, C D (1993) Studies of the phylogeny and classification of long-tongued bees (Hymenoptera: Apoidea). *University of Kansas Scientific Bulletin* 55: 123–173.

SERRAO, J E (2001) A comparative study of the proventricular structure in corbiculate apinae (Hymenoptera, Apidae). *Micron* 32: 379–385.

SERRAO, J E (2005) Proventricular structure in solitary bees (Hymenoptera: Apoidea). *Organisms Diversity and Evolution* 5: 125–133.

TOLLNER, E W (1991) X-ray computed tomography applications in soil ecology. *Agriculture, Ecosystems and Environment* 34: 251–260.

WILLE, A (1979) Phylogeny and relationships among the genera and subgenera of the stingless bees (Meliponinae) of the world. *Revista de Biologia Tropical* 27: 241–277.